

Cell Nanocoating for Rapid Microbial Screening

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Abstract

Microbial contamination is a global challenge facing not only the food and pharmaceutical industries but also water safety and clinical hygiene control. Traditional microbial identification methods suffer from costly and time-consuming processes. Rapid microbial screening assays overcome these limitations, however, very few rapid microbial screening assays are available on the market. Rapid microbial screening refers to the detection of the total microbial load in samples without specifying the strains or species. The primary goal of this study is to develop a rapid microbial screening assay that yields accurate and quantifiable results in less than 30 min.

Nanocoating of single microbial cells with gold nanostructures can confer optical, electrical, thermal and mechanical properties to the outer layers of microorganisms, thus enabling new avenues for their control, study, application and detection. Cell nanocoating is often performed using layer-by-layer (LbL) deposition of functional materials. LbL is time-consuming and relies on nonspecific electrostatic interactions, which can be unstable in adverse sample environments and limit its potential applications for microbial diagnostics. This thesis shows that by taking advantage of surface molecules densely present in the outer membrane layers, cell nanocoating with gold nanoparticles can be achieved within seconds.

The objective of this thesis is to develop a rapid microbial detection system by coating the densely populated surface molecules on the outer layer of microbes with gold nanoparticles. These surface molecules include disulfide bond-containing (DsbC) proteins and chitin, which can be activated with a simple one step process. This activation leads to

subsequent interactions with gold nanoparticles that allow for specific microbial screening and quantification of bacteria and fungi within 5 and 30 min respectively. The transduction methods such as plasmonics and fluorescence offers a limit of detection below 35 cfu.mL⁻¹ for bacteria and 1500 cfu.mL⁻¹ for fungi using a portable reader.

Table of Contents

	Page
Acknowledgement	i
Abstract	ii
List of Tables	v
List of Figures	vi
Chapter 1. Introduction.....	1
Chapter 2. Literature Review	5
2.1 Methods for Microbial Identification.....	5
2.1.1 Enumeration Assays	5
2.1.2 Immunoassays.....	6
2.1.3 DNA-based Assays	7
2.2 Methods for Microbial Screening	8
2.2.1 Adenosine Triphosphate Bioluminescent Assay.....	8
2.2.2 Limulus Amebocyte Lysate Assay	10
2.3 Cell Nanocoating	11
2.4 Rapid microbial screening with Nanocoating.....	13
2.4.1 Gold Nanoparticles in Biosensing	13
2.4.2 Cell Nanocoating with Gold Nanoparticles	15
Chapter 3. Experimental Section	16
3.1 Materials and Instrumentation	16
3.2 Preparation of Gold Nanoparticles.....	16
3.3 Preparation of the Microbial Suspensions and Cell Coating.....	17
3.4 Cell Nanocoating and Microbial Screening	18
Chapter 4. Results and Discussion	19
4.1 Disulfide-bond-containing protein layers as surface markers for microbial screening	19
4.2 Chitin layers as surface markers for fungal screening	23
4.3 Plasmonic and fluorescence detection assays	34
Chapter 5. Conclusion and Prospectives	42
Bibliography	44

List of Tables

	Table
Table 6.1 Microorganisms tested for plasmonic cell nanocoating	22

List of Figures

Figure 1. Plasmonic cell nanocoating using Dsbc surface protein layers.....	20
Figure 2. Plasmonic cell nanocoating using chitin	25
Figure 3. Effect of the deacetylation <i>Lactobacillus delbrueckii</i> and <i>Mucor</i> <i>circinelloides</i> on the assembly of AuNPs	26
Figure 4. SEM, TEM and EDX image of microbial cells coated with AuNPs.....	27
Figure 5. SEM images of microbial cells coated with AuNPs at higher magnification	28
Figure 6. TEM images of microbial cells coated with AuNPs	30
Figure 7. Stability of the microbial Dsbc surface protein layer	32
Figure 8. SEM images of bacteria <i>E. coli</i> pili coated with AuNPs.....	33
Figure 9. Schemes of the screening assays based on plasmonic cell nanocoating	34
Figure 10. Rapid microbial screening of microbes by plasmonic cell nanocoating	36
Figure 11. Effects of the reduction of the concentration of the AuNPs on the visual reading of the microbial load	41

CHAPTER 1 Introduction

Microbial contamination is a worldwide challenge facing not only food industry, but water safety and clinical hygiene control as well. The United States Centers for Diseases Control and Prevention (CDC) reported that about 9.4 million people become ill due to 31 major foodborne pathogens and 1,351 people die annually (Scallan et al., 2011). World Health Organization (WHO) also estimated that drinking water that is contaminated due to poor sanitation causes 502,000 deaths every year (WHO, 2017). Over the years, there are significant improvements in microbial detection methods for microbial control, and various enumeration methods, DNA-based and immune-based assays, and other biosensors have been developed. However, these detection methods are generally based on specific detection, where long incubation time and sample pretreatments prevent these methods from rapid tests at large scale. Plate counting suffers from prolonged incubation period; high specificity of DNA-based and immunoassays can turned to be downside, as almost half of the microorganisms related to all disease outbreaks are not identified (Kaaden & Czemy, 1997). Thus, a microbial detection method that is rapid, cheap and applicable for most microorganisms can be very helpful to evaluate the microbial content in the samples.

Rapid microbial screening refers to the detection of the presence of a certain type of microorganism without specifying the strains or species, which is an important aspect in microbial diagnostics. Not only can it save time and resources in decision making before engaging in costly and time-consuming microbial identification, but it can also meet

numerous industrial needs where the main concern is the presence of a certain class of microorganisms in the product regardless of the species (bacteria contamination in pharmaceuticals, fungal contamination in some food products, or situations where the microbial load is a relevant clinical indicator of infection or contamination).

Very few rapid microbial assays are available on the market nowadays. These been namely, the *Limulus* Amebocyte Lysate (LAL) test for the screening of gram-negative bacteria and endotoxins (Seiter & Jay, 1980) and the adenosine triphosphate (ATP)-bioluminescence assay largely used for the evaluation of biocontamination, but not specifically for the presence of microorganisms (Bottari, Santarelli, & Neviani, 2015). Both tests can generate false positive results due to weak specificity caused by indirect detection and suffer from variability issues across instruments. The limit of detection ranges between 10^3 and 10^5 cfu.mL⁻¹ (Fulford, Walker, Martin, & Marsh, 2004; Omidbakhsh, Ahmadpour, & Kenny, 2014). Regarding fungi (yeast and mold), there is currently no available rapid detection test, and current methods are based on cell plating and incubation for a few days followed by colony count.

Since the first report on cell nanocoating two decades ago (Davis, Burkett, Mendelson, & Mann, 1997), diverse applications of cell nanocoating have been proposed including biotemplating for hierarchical nanoparticle assembly (Z. Li, Chung, Nam, Ginger, & Mirkin, 2003), environmental remediation (Konnova, Lvov, & Fakhrullin, 2016), nanoparticle delivery (Däwlätsina, Minullina, & Fakhrullin, 2013), and the fabrication of hybrid bioelectronic devices (Vikas Berry & Saraf, 2005). Microbial cell nanocoating has so far mainly been achieved using layer-by-layer deposition of

polyelectrolytes (Rawil F. Fakhrullin & Lvov, 2012; Park et al., 2014), either functionalized or intercalated with the desired inorganic nanostructures (Vikas Berry & Saraf, 2005; Rawil F Fakhrullin, García-Alonso, & Paunov, 2010; Rawil F. Fakhrullin et al., 2009; Konnova et al., 2016; Sung Ho Yang et al., 2009). Other less common methods include surface-induced ion reduction (biomineralization) on bacteria (Reith, Rogers, McPhail, & Webb, 2006) or growth-driven assembly on fungi, a process that typically requires a long time to complete (Z. Li et al., 2003; Sugunan, Melin, Schnürer, Hilborn, & Dutta, 2007). LbL deposition relies on electrostatic interactions between the deposited materials and the microbial surface. Such interactions are not specific to microorganisms, which explains why inorganic cell nanocoating with gold nanoparticles (AuNPs) and other materials has so far not been explored for microbial screening or detection.

In this work, we introduce a novel concept for microbial screening based on selective cell nanocoating. Besides antigenic molecules that are specific to the microbial species or strains and that are usually targeted in immunoassays, microorganisms exhibit surface molecules that are characteristic of the microbial class. These surface molecules include chitin in fungi (Bartnicki-Garcia, 1968), hydrophobin in filamentous fungi (Linder, Szilvay, Nakari-Setälä, & Penttilä, 2005), lipopolysaccharides in gram-negative bacteria (Lüderitz et al., 1982). and lipoteichoic acid in gram-positive bacteria (Weidenmaier & Peschel, 2008) . Targeting the molecules that are populated densely on the surface of the microorganisms to induce cell nanocoating would provide the necessary specificity for target microbes from background cells. The process can then enable rapid microbial screening without using antibodies or other bioreceptors, which reduces costs and

accelerating detection. Thus, we hypothesize that using plasmonic AuNPs as a coating material would offer a rapid, versatile and sensitive transduction system (Anker et al., 2008). The object of this study is to develop a rapid microbial screening assay by coating the surface of microorganisms with AuNPs via the surface molecules, and develop transduction systems to accurately quantify the number of cells in sample.

CHAPTER 2 Literature Review

Microbial detection methods can be classified as either microbial identification methods or microbial screening methods. Microbial identification is the specific detection of microorganisms to identify the strains or species. In contrast, microbial screening allows the analysis of total microbial load instead of specifying species or strains. In this section, major microbial detection assays that are commonly used in research or industrial settings are introduced and compared.

2.1.Methods for Microbial Identification

2.1.1. Enumeration Assays

The most traditional and the most time-consuming microbial detection method is cell counting on nutritive media or selective media, which generally takes about 12-48 hours for bacteria to several weeks for fungi because of the growth capabilities of the cultures. In addition to the long culturing time, some targeted microorganisms may not be isolated or cultured, which leads to false negative results (Davey & Kell, 1996). In contrast to the various screening methods for bacteria, few assays are available for fungi (yeast and mold), and the most prevalent detection test still relies on colony counting after several days or weeks required for cell growth.

Flow cytometry is another enumeration method for microbial screening. In this system, small volumes of microbial samples are driven into the system with a laminar flow before encountering the focused light beam. Light scattering can be measured in the system according to the cell's shape and sizes, and fluorescence intensity can be sorted into different channels and detected based on the dyes used. This method avoids sample

isolation and future culturing, and can be used for both qualitative and quantitative cell detection and differentiation (Laplacebuihe, Hahne, Hunger, Tirilly, & Drocourt, 1993). Gunasekera et al. has reported the detection limit of microbial contamination in milk to be smaller than 10^4 bacteria per milliliter of milk using flow cytometry within 45 to 60 minutes. Although this technique allows direct detection of individual cells, it fails to detect small amount of microorganisms with high accuracy due to the limitations on the volumes of the sample per test (Gunasekera, Attfield, & Veal, 2000).

2.1.2. Immunoassays

Immunoassays for microbial detection rely on the highly specific interaction between antibodies and antigens. Enzyme linked immunosorbent assay (ELISA) combines an immunoassay with an enzyme assay. Target antigens in the sample will bind to the antibodies immobilized on a surface of 96-well microtiter plate. After incubation, unbound materials are washed away and a secondary antibody targeting the antigen is added to form a “sandwich” structure. Followed by the addition of a secondary enzyme-labelled antibody which will bind to the previous antibody, the unbound secondary antibodies will be rinsed away. The final step requires the addition of a substrate for the enzyme to generate signals for detection (Jasson, Jacxsens, Luning, Rajkovic, & Uyttendaele, 2010).

Immunoassays have been applied to various food samples like seafood and poultry products for microbial control and allergen tests. The total time for ELISA detection is about 4 hours, but the sample enrichment can vary significantly from 5 hours to 5 days depending on the sample matrix with a detection limit ranging from 10^3 - 10^5

CFU.mL⁻¹ (Crocì, Delibato, Volpe, & Palleschi, 2001; B. K. Kumar et al., 2011; Lilja & Hänninen, 2001). The major drawback for the immunological-based detection is the prolonged sample enrichment time, and relatively low sensitivity (Velusamy, Arshak, Korostynska, Oliwa, & Adley, 2010).

2.1.3. DNA-based Assays

Polymerase Chain Reaction (PCR) is one of the most widely used molecular-based technique to detect foodborne bacterial pathogens. This approach has been available for about 30 years and can literally detect a single bacterial cell by amplifying the target DNA sequence with a three-step cycle process (Batt, 2007). One PCR cycle includes denaturation of double-stranded DNA to two single-stranded DNA sequences under high temperature (95°C), then forward and reverse primers will anneal to the template strands at around 50-65°C, followed by elongation process whereby free deoxyribonucleotides complementary to the template strand are added by DNA polymerase in the 5'-3' direction at 72°C. The cycle continues until copy number of DNA generated during amplification is sufficient for detection, allowing for the products to be visualized by gel electrophoresis (Bartlett & Stirling, 2003). The specificity, accuracy and sensitivity features of PCR approach have made it very competitive among the available detection methods. Kumar et al. have showed that PCR assay was more sensitive than conventional culturing and immune-based assay (ELISA) when detecting *Salmonella typhimurium* in seafood (R. Kumar, Surendran, & Thampuran, 2008). However, PCR can also detect non-viable microbes by only targeting the genetic material, which makes it hard to differentiate live and dead cells (Josephson, Gerba, & Pepper, 1993). In addition,

the total time required for sample preparation, PCR cycles and gel electrophoresis makes this method not ideal for rapid detection.

Quantitative real-time PCR can be a good alternative approach in this regard, as it eliminates the need for post-PCR processing, which can reduce the chances for post-PCR contamination and save time (Valasek & Repa, 2005). False negative PCR results can occur for both assays if there is inhibitory component (phenolic compounds) in food samples as well as the carry-over of background DNA contamination, which results in the need for more extensive sample preparation (Bricker, 2002; David & Relman, 1999; Wilson, 1997).

2.2. Microbial Screening methods

The other track in microbial detection is microbial screening, which allows the analysis of total microbial load instead of specifying species or strains. As discussed previously, microbial screening is preferred as it save time and resources in decision making, and it works best when only a certain class of microorganisms in the product (regardless of the species) is the main concern. The next section introduces some of the few rapid microbial screening assays available in the market.

2.2.1. Adenosine Triphosphate (ATP) Bioluminescent Assay

Adenosine Triphosphate (ATP) Bioluminescent Assay was used to detect microbial content in food back to 1970s (Sharpe, Woodrow, & Jackson, 1970). Based on the oxidative decarboxylation of luciferin to oxyluciferin, the reaction is catalyzed by luciferase and is driven by the energy released from ATP hydrolysis. The product of this reaction also includes light that can be measured with a luminometer for quantification.

Since the bioluminescence reaction can be initiated with a minimal amount of ATP, it is possible that the assay can be used to detect low concentrations of microbes in contaminated samples (Lyman & DeVincenzo, 1967). The limit of detection for current commercial kits varies greatly from 10^2 to 10^5 cfu.mL⁻¹ due to differences between sample matrices and instrument sensitivities (Bottari et al., 2015; Fulford et al., 2004; Omidbakhsh et al., 2014).

A significant advantage of this method over the previous assays is that the total time of the assay is short, even less than 30 minutes (Bottari et al., 2015; Hawronskyj & Holah, 1997). However, the fact that ATP is present in both non-microbial (somatic cells) and microbial cells can cause false positive results, and thus requires sample pretreatment to extract target intracellular ATP from microbial cells. In addition, a standard calibration curve for microbial quantification is hard to develop, as the intracellular ATP content is different between the species, between the cells of the same species, and even between different growing stages of the same cell (Bottari et al., 2015). When this system is applied to real-world samples, the disinfectants and cleansing agents that are regularly used in food industry and clinical settings can also act as ATP-releasing agents, and thus affect the accuracy of the bioluminescence test (Green, Russell, & Fletcher, 1999; Lappalainen et al., 2000). There are currently lots of commercial products to do bacterial ATP-based bacterial test on surfaces. In these products, swabs are used to collect samples from surfaces and are then suspended in testing media for measurement, and samples in solution can be directly applied to the system (Hawronskyj & Holah, 1997). This assay has also been included in Hazard Analysis and Critical Control Points

(HACCP) measurements, which is the system recommended by the Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) for food production plants to implement for hazard analysis and control for food safety (USDA, 2000) (Osimani, Garofalo, Clementi, Tavoletti, & Aquilanti, 2014).

2.2.2. The Limulus Amebocyte Lysate Assays

The Limulus Amebocyte Lysate (LAL) test is for the screening of gram-negative bacteria and endotoxins. When amebocyte lysate extracted from the *Limulus Polyphemus* (horseshoe crab) in the presence of endotoxin on the surface of gram-negative bacteria, gel formation will be observed (Levin & Bang, 1964; Seiter & Jay, 1980). Endotoxin can trigger a cascade reaction of serine proteases, which results in the formation of a gel clot (Ding & Ho, 2010). This test has been implemented in the pharmaceutical industry as an alternative assay to replace the rabbit pyrogen test because of its sensitivity and accuracy (Devleeschouwer, Cornil, & Dony, 1985). The assay has also been applied to endotoxin detection for water and food quality control. Jay et al. has reported using the test to measure the endotoxin content in ground beef (Jay, Margitic, Shereda, & Covington, 1979).

Over the years, the LAL test has been improved and simplified for commercial use for protein detection. However, results for LAL test are mainly reported as mass-based, which makes quantitation of cells difficult due to the variations in the amount of reactive endotoxins on the microbial surface under same preparation (Jay et al., 1979). The cascade reactions of several enzymes are very sensitive to pH, protein content and the presence of inhibitors in the sample, which affects the reproducibility of the assay

(Novitsky, 1998). Besides that, over-harvesting of horseshoe crabs for the fishery and research purposes within biomedical sciences has also decreased the population of horseshoe crabs to potential extinction (Widener & Barlow, 1999).

The assays mentioned above are some of the most widely used methods, but there are other developed methods like optical sensors based on surface plasmon resonance (SPR) effects, Fourier transform infrared spectroscopy (FT-IR), and Raman spectroscopy (Al-Holy, Lin, Cavinato, & Rasco, 2006; Schmilovitch et al., 2005) (Taylor et al., 2006). Other sensors, like piezoelectric sensors are mass based, but these assays are less commonly used for commercial purposes (Su & Li, 2005).

2.3. Cell nanocoating

Cell coating, or cell encapsulation, is defined as the deposition of a semipermeable membrane on the cell surface (Chang, 1964). Coating isolates the cell from its surroundings as a physical barrier while maintaining the inside cell viability and permeability (Uludag, De Vos, & Tresco, 2000). This technique can have significant therapeutic applications as it can protect transplanted cells from immune rejections without suppressing the immune system (Sun, Ma, Zhou, Vacek, & Sun, 1996).

Cell nanocoating refers to the application of an ultra-thin film composed of nanomaterials (<100nm) on the cell surfaces (Park et al., 2014). This research field has a relatively short history, but has made impressive development over the years (Park et al., 2014). The major microbial cell nanocoating strategy is layer-by-layer (LbL) deposition of polyelectrolytes, nanoparticles or proteins, by which layers of charged material are deposited on the oppositely charged cell surface through binding by electrostatic

interactions (Rawil F. Fakhrullin & Lvov, 2012; Park et al., 2014). Cell nanocoating can be also achieved by some less common methods. Non-electrostatic LbL cell coating on yeast was achieved using hydrogen bonding (Kozlovskaya et al., 2011). Biomineralization is another example of cell nanocoating based on surface-induced ion reduction of materials on bacteria (Reith et al., 2006). Growth-driven assembly of gold nanoparticles on microbial cell surface has also been reported; this method is based on the concept which fungal cells are cultured in a colloidal medium containing unreacted precursors for gold nanoparticle synthesis. In the medium, absorption of nutrients drives and assembles the nanoparticles on the cell surface (Z. Li et al., 2003; Sugunan et al., 2007).

The advantages of cell nanocoating made this technique a good alternative to the traditional cell adhesion or surface coating. The increased surface area to volume ratio due to nanoparticle sizes makes the coated surface more available for chemical manipulation at the single cell level (S. H. Yang, Hong, Lee, Ko, & Choi, 2013). Moreover, there are numerous combinations of coating materials available that allow functionalizing the microbial cell for various applications (Rawil F. Fakhrullin & Lvov, 2012). One of the useful application is biotemplating, where cells serve as destroyable templates and will be sacrificed after coating, thus forming hollow capsules with ordered nano-structure (hierarchical nanoparticle assembly) (Z. Li et al., 2003).

Another major field of single cell nanocoating is artificial spores. Fungal cells are known to be dormant in a state called “sporulation” to protect cells and survive in nutrient deprived, harsh environments. Since ultraviolet (UV) radiation, extreme pH

conditions, osmotic pressure, dehydration and other physical and chemical stresses can greatly impact the growth and viability of cells, protective endospores can enable the cells to persist longer (McKenney, Driks, & Eichenberger, 2013). In the case of artificial spores, layers of nanostructure coating around living cells can mimic the functions of sporulation, and provide additional protection while maintaining selective permeability and cell viability (Rawil F. Fakhrullin et al., 2009). Moreover, the shell can be functionalized to facilitate cell interaction with controlled degradability, so that the cell-coating structure can be manually controlled when the exposure of the original cell is needed (Hong, Hyea Ko, & Choi, 2014).

Biosensors can incorporate nano-coated cells into the detection system. Some studies have reported the use of bacteria cells coated by nanoparticles as part of the hybrid bioelectronics devices due to the enhanced conductivity after coating (V. Berry, Gole, Kundu, Murphy, & Saraf, 2005; Vikas Berry & Saraf, 2005). Gold nanorod coated cancer cells can be visualized in the near IR range and allow visualization under microscope (X. Huang, El-Sayed, Qian, & El-Sayed, 2006). Genetically modified green fluorescent protein (GFP) reporter yeasts have been studied for toxins or other chemical detection after being functionalized with magnetic nanoparticles (García-Alonso et al., 2011).

2.4.Rapid microbial screening with nanocoating

2.4.1. Properties of Gold nanoparticles in biosensing

AuNPs have been studied extensively for biomolecular sensing including colorimetric, fluorometric, electrochemical, and plasmon resonance based sensing (Saha,

Agasti, Kim, Li, & Rotello, 2012). Gold nanoparticles are characterized to have Surface Plasmon Resonance (SPR), which is caused by the resonance between the incident light and the collective oscillation of the surface electrons of nanoparticles (Ghosh & Pal, 2007). When gold particles are small in the nano-scale, the increased surface area to volume ratio due to nanoparticle sizes makes the coated surface more available for chemical manipulation at the single cell level the strong absorption of green light in the visible range at about 520 nm leads to a ruby red color in solution (Ghosh & Pal, 2007). If gold nanoparticles aggregate, a red shift (520nm-650 nm) in the surface plasmon band will result in color change from ruby red to dark blue due to plasmon coupling effects between particles (Srivastava, Frankamp, & Rotello, 2005). Since the color change can be visualized, AuNPs can be used for colorimetric sensors. Currently, AuNPs-based colorimetric assay has been applied to detect toxic metal ions, DNA, proteins and cells (Aili, Selegård, Baltzer, Enander, & Liedberg, 2009; Elghanian, Storhoff, Mucic, Letsinger, & Mirkin, 1997) (Guo, Wang, Qu, Shao, & Jiang, 2011; Medley et al., 2008) .

Fluorescence quenching is often observed when fluorophores are added to AuNPs. Molecular beacon, a FRET-based system for DNA sensing, is based on this principal (Saha et al., 2012). The fluorophore and the AuNP are brought close to each other within a few angstroms by the hairpin structure of the single strand DNA, and AuNP quenches the fluorescence of the fluorophore. If the target (single-stranded DNA) is hybridized with the hairpin structure, the conformation changes and separates the fluorophore and the AuNP far from each other to restore the fluorescence (Dubertret, Calame, & Libchaber, 2001). This approach has been used to detect RNA, DNA, amino acids and

metal ions (Dubertret et al., 2001; T. Huang & Murray, 2002; Lin, Chang, & Tseng, 2010; Prigodich et al., 2009). El-Sayed et al. have also reported a possibility to detect cancer cells by coating cancerous cell surfaces with oligonucleotide functionalized gold nanoparticles (El-Sayed, Huang, & El-Sayed, 2005) .

2.4.2. *Single cell nanocoating with AuNPs*

As mentioned in the previous section, there are significantly increasing number of studies about the use of nanoparticles in nanocoating for cellular modification. Gold nanoparticles are among the most promising tools and has been applied to construct hierarchical assembly, electrically conducting devices and sensor development (V. Berry, Rangaswamy, & Saraf, 2004; X. Huang et al., 2006; Z. Li et al., 2003). Among the various ways that gold nanoparticles can be coated on cell surfaces, LbL is still the dominant strategy. However, LbL deposition based on electrostatic interactions cannot differentiate microorganisms from somatic cells in the matrix, which requires cell purification to avoid false positive responses. AuNPs can also be functionalized with antibodies or oligonucleotides, but these types of coating methods are already time consuming before further applications (W. Li et al., 2015) (Keeney et al., 2015) (El-Sayed et al., 2005). Thus, specifically coating target microbes with AuNPs while utilizing the molecules may offer a rapid, versatile and sensitive transduction system (Anker et al., 2008). In the rest of the paper, microbial screening methods that detect target microbes coated with AuNPs via the surface molecules and transduction systems that are rapid and sensitive will be discussed.

CHAPTER 3 Experimental Section

3.1. Materials and instrumentations

Gold (III) chloride trihydrate, trisodium citrate dehydrate, trisodium citrate dehydrate, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), sodium hydroxide (NaOH), rhodamine 6G, and 2-mercaptoethanol (BME) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All growth media for microbial culture were purchased from Aldrich-Sigma. All reagents were used as received unless otherwise specified. All microorganisms were purchased from the American Type Culture Collection (ATCC).

All fluorescence experiments were carried out with the GloMax® MultiJR fluorometer with excitation wavelength of 525 nm. The gold nanoparticles and optical density (OD) of microbes were characterized using a UV-visible spectrophotometer (Shimadzu UV-1800, Shimadzu Corp., USA). Centrifugation was performed with microcentrifuge (MiniSpin Plus, Eppendorf™, USA). Extrusion of bacteria for fluorescence assays was achieved using the mini-extruder kit from Avanti Polar Lipids, Inc., USA. Raman and surface-enhanced Raman scattering analysis was performed using Witec Alpha 300 R Confocal Raman microscope with UHTS300 spectrometer and DV401 CCD detector.

3.2. Preparation of gold nanoparticles

Gold nanoparticles were prepared following the protocol developed in the lab based on a modification of Turkevich's methods (Bui, Ahmed, & Abbas, 2015; Grabar, Freeman, Hommer, & Natan, 1995; Turkevich, Stevenson, & Hillier, 1951). Briefly, 1 mM of HAuCl₄ solution was boiled on a hot plate for 5 min, followed by adding 10 mL

of briefly preheated 38.8 mM sodium citrate solution. After 30 s stirring, the solution was taken off from the hot plate and cooled to room temperature. The color of the final solution is ruby red and has a strong absorption peak at 520 nm as measured by a UV-vis spectrophotometer. The size of AuNPs was characterized to be 12 ± 2 nm in diameter using transmission electron microscopy (TEM, FEI Technai T12).

3.3.Preparation of the microbial suspensions and cell nanocoating

Bacteria Samples:

Escherichia coli Castellani and Chalmers (ATCC 25922) were grown on tryptic soy agar (TSA) and *Lactobacillus delbrueckii* subsp. *bulgaricus* were grown on the De Man, Rogosa and Sharpe (MRS) agar at 37 °C overnight and store at 4 °C until use. Before use, the concentration of the microbial suspension was evaluated by measuring the optical density, and a serial of ten-fold dilution was performed to prepare different microbial concentrations from 10 to 10^8 cfu.mL⁻¹. The microbial concentrations were confirmed using the BD Accuri™ C61 flow cytometer (BD Biosciences, USA), a hemacytometer and plate counting methods.

Fungi:

Saccharomyces cerevisiae var. *boulardii* (ATCC MYA-796™) were grown in the YM broth at 30 °C with 200 rpm shaking overnight. *Mucor circinelloides* (ATCC® MYA-3787™) were grown in the potato dextrose agar (PDA) media at 25 °C. All samples were then centrifuged twice for 5 minutes at 10,000 g to remove media and suspended in water. The microbial cultures were stored at 4 °C and reactivated at growth temperature before use. For deacetylation, 50 % (w/v) NaOH solution was added to

samples at equal volume ratio. The mixture was vortexed well and rotated at 30 rpm in a tube revolver for 25 minutes. Then, the solution was centrifuged at 6,700 rpm for 5 minutes and re-suspended in nanopure water. The solution pH was further adjusted to 7.0 using 1M HCl solution. The microbial concentration was evaluated and different dilutions were prepared. The microbial concentrations were confirmed using the BD Accuri™ C61 flow cytometer (BD Biosciences, USA), a hemacytometer and plate counting methods.

3.4. Cell nanocoating and microbial screening

For microbial screening using plasmonic detection (or UV-vis spectroscopy), 400 μ L of microbial sample was mixed with 80 μ L of 10 mM TCEP solution and incubated for 5 min. Then, 400 μ L of AuNP solution was added to, and the sample absorbance is immediately measured at 600 nm using a UV-visible spectrophotometer.

For fluorescence detection, the reduction of bacteria for 5 min is followed by removing TCEP from the sample using a mini-extruder. This step is required because TCEP interferes with the fluorescence signal of Rhodamine 6G. Then, 120 μ L of the AuNP solution was added to 850 μ L of reduced microbial samples. Then, 30 μ L of freshly prepared 1 mM solution of Rhodamine 6G was added to the mixture and the fluorescence intensity was measured over time with a 3s interval for a period of 3 minutes. Control sample measurements were performed using non-reduced or non-deacetylated microbial samples and AuNP-TCEP samples as controls.

CHAPTER 4 Results and Discussion

4.1. Disulfide-bond-containing (Dsbc) protein layers as a surface marker for microbial screening

Initially, the goal for the study is to specifically detect filamentous fungi in environmental samples. Knowing that these fungi and spores are surrounded with a layer of hydrophobins which are surface proteins that contain 4 disulfide bonds (Linder et al., 2005), we hypothesized that mixing the fungal suspension with a reducing agent would reduce the disulfide bonds, yielding free reactive thiol groups. The subsequent addition of gold nanoparticles (AuNPs) would cause the nanoparticles to interact with the thiol groups and spontaneously form a thin monolayer coating on the fungal surface (**Figure 1a**). At high microbial concentrations, the interaction would result in a visible color change of the suspension from red (single dispersed nanoparticles) to dark blue (nanoparticle assembly), caused by a plasmonic coupling of localized surface plasmon resonance in gold nanoparticles (A. Abbas, Kattumenu, Tian, & Singamaneni, 2013; Bui et al., 2015; Ghosh & Pal, 2007).

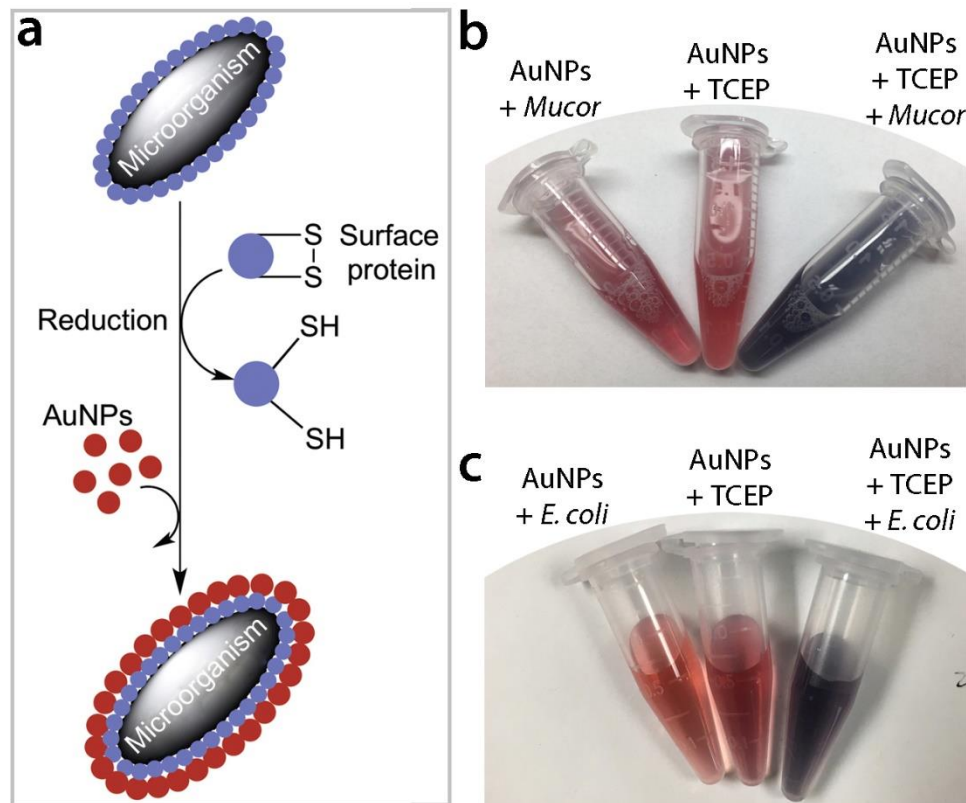


Figure 1. Plasmonic cell nanocoating using DsbC surface protein layers. (a) Scheme of plasmonic cell nanocoating of microorganisms by reducing the disulfide-bond containing proteins on the microbial surface. (b) and (c) Pictures of AuNP solution mixed with either fungi (*Mucor*) or bacteria (*E. coli*) showing the change in color after addition of a reducing agent TCEP.

To demonstrate this concept, a fungal suspension of *Mucor circinelloides* was prepared with a final concentration of 10^8 cfu.mL⁻¹, and tris(2-carboxyethyl)phosphine (TCEP) was used as reducing agent. As expected, reduction of the fungal suspension with TCEP for one minute followed by the addition of AuNPs results in an immediate color change from red to dark blue (**Figure 1b**). Control experiments containing AuNPs and fungi or AuNPs with TCEP did not show any change in color of AuNPs. Replacing TCEP with another reducing agent such as 2-mercaptoethanol (BME) resulted in similar aggregation.

An interesting bioinformatics study by Dutton et al, predicted the formation of disulfide bond in the cell envelope across different bacterial species (Dutton, Boyd, Berkmen, & Beckwith, 2008) . Based on this prediction along with other studies describing the important role of disulfide bonds on microbial cell stability and functionality,(Heras et al., 2009; Hogg, 2003) we hypothesized that DsbC proteins may be ubiquitous in other microorganisms, and thus can be used for rapid microbial screening using cell nanocoating.

To test this hypothesis, we decided to extend the same experiment previously performed on *Mucor circinelloides*, to non-filamentous fungi *Saccharomyces boulardii*, gram-negative bacterium *Escherichia coli*, and gram-positive bacteria *Lactobacillus delbrueckii* subsp. *bulgaricus*. Interestingly, the same reaction and color change was also observed with the non-filamentous fungus and with both gram-negative and gram-positive bacteria (**Figure 1c**). Twenty species of bacteria, yeasts and molds were tested and all showed a positive reaction with gold nanoparticles after reduction with TCEP (**Table 1**). These results reveal the existence of reducible moieties with high affinity to gold surface. Surface-enhanced Raman analysis of the coated microorganisms revealed a peak at 317 cm^{-1} assigned to Au-S bonds (Varnholt et al., 2014), thus suggesting the existence of thiol-containing molecules on the microbial surface (**Figure 1d**).

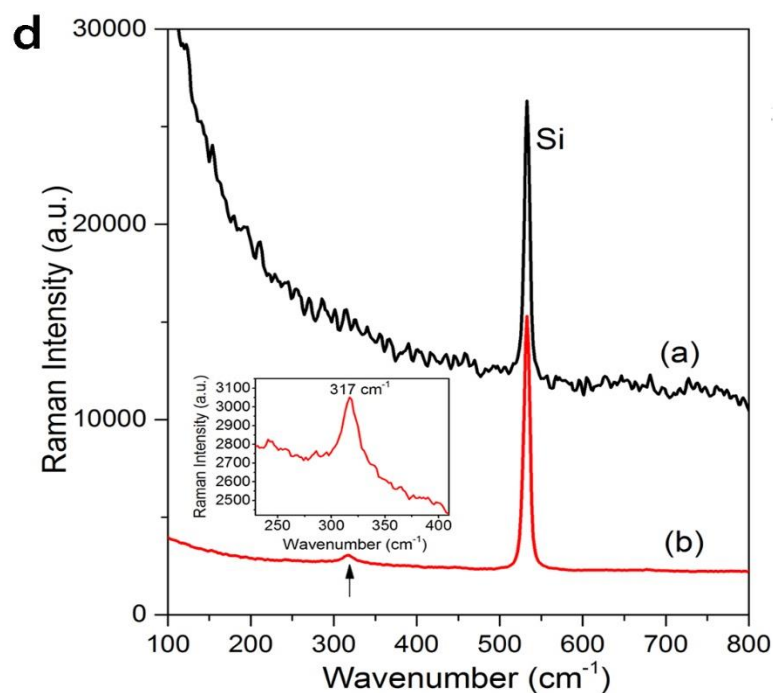


Figure 1. (d) Surface-enhanced Raman spectra of a mixture of fungi (*Mucor*) and AuNPs before (spectrum *a*) and after (spectrum *b*) addition of a reducing agent TCEP. The peak at 317 cm^{-1} is assigned to Au-S bonds.

Table 1

Types of Microorganisms	Names
Yeasts	<i>Saccharomyces boulardii</i> , <i>Torulaspora delbrueckii</i> , <i>Rhodotorula mucilaginosa</i> , <i>Cryptococcus carnescens</i> , <i>Candida kefyr</i> , <i>Ceratocystis fagacearum</i>
Molds	<i>Penicillium commune</i> , <i>Aspergillus niger</i> , <i>Cladosporium</i> <i>spp.</i> , <i>Penicillium roqueforti</i> , <i>Radopholus similis</i>
Dimorphic fungi	<i>Mucor circinelloides</i>

Gram-positive bacteria	<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> , <i>Lactobacillus acidophilus</i> , <i>Lactobacillus casei</i> , <i>Lactobacillus gasseri</i> , <i>Methicillin-resistant Staphylococcus aureus (MRSA)</i> , <i>Listeria monocytogenes</i>
Gram-negative bacteria	<i>Escherichia coli</i> , <i>Salmonella typhimurium</i>

Table 1. 20 microorganisms tested for plasmonic cell nanocoating: The microorganisms in the table yielded a positive reaction when treated with TCEP and mixed with gold nanoparticles

For macroscopic organisms, such as animal and plant tissues, disulfide bonds are generally present in lysosomal proteins, secretory proteins, and in some membrane proteins (Regeimbal & Bardwell, 2002). However, their presence in the outer layers does not seem to be ubiquitous in a way that can cause nanoparticle assembly with short-range plasmonic coupling. In fact, the plasmonic coupling that causes a change in color vanishes exponentially with the increasing distance between the nanoparticles, and becomes weak or inexistent beyond 20 nm distance as was previously reported (Abdenmour Abbas, Fei, Tian, & Singamaneni, 2013). While further study and characterization of these reducible surface molecules is an interesting endeavor, this work focuses on using these molecules for rapid diagnostic purposes

4.2.Chitin layers as a surface marker for fungal screening.

In addition to total microbial load, it is useful in rapid screening to know the microbial type present in the sample. Here, we show how this can be achieved by taking fungi as an example. To enable specific detection of fungi in a multispecies microbial

suspension, it is important to first identify a surface molecule that is specifically present on fungi. Chitin is a rigid polysaccharide-based three-dimensional network, unique to fungal cell walls and the exoskeletons of arthropods (Latgé, 2007). Hence, chitin could be considered as a specific marker for fungal screening in a complex microbial sample. Similar to the disulfide bonds, chitin requires activation to enable its interaction with gold nanoparticles. The activation is obtained by converting fungal chitin into chitosan through a deacetylation process by incubating the sample with 50% sodium hydroxide for 30 min. The reaction yielded free reactive primary amine groups at the fungal surface. The subsequent addition of AuNPs to deacetylated fungi spontaneously results in very dense and highly stable cell nanocoating (**Figure 2a**). The same color shift from red to dark blue can be seen in the fungal suspension at high concentrations (**Figure 2b and 2c**). Although the cell wall in gram-negative bacteria contains *N*-Acetylglucosamine (a monomeric unit of the polymer chitin), the deacetylation of both gram-negative and gram-positive bacteria does not yield any color change, indicating that the test is specific to fungi (**Figure 3**).

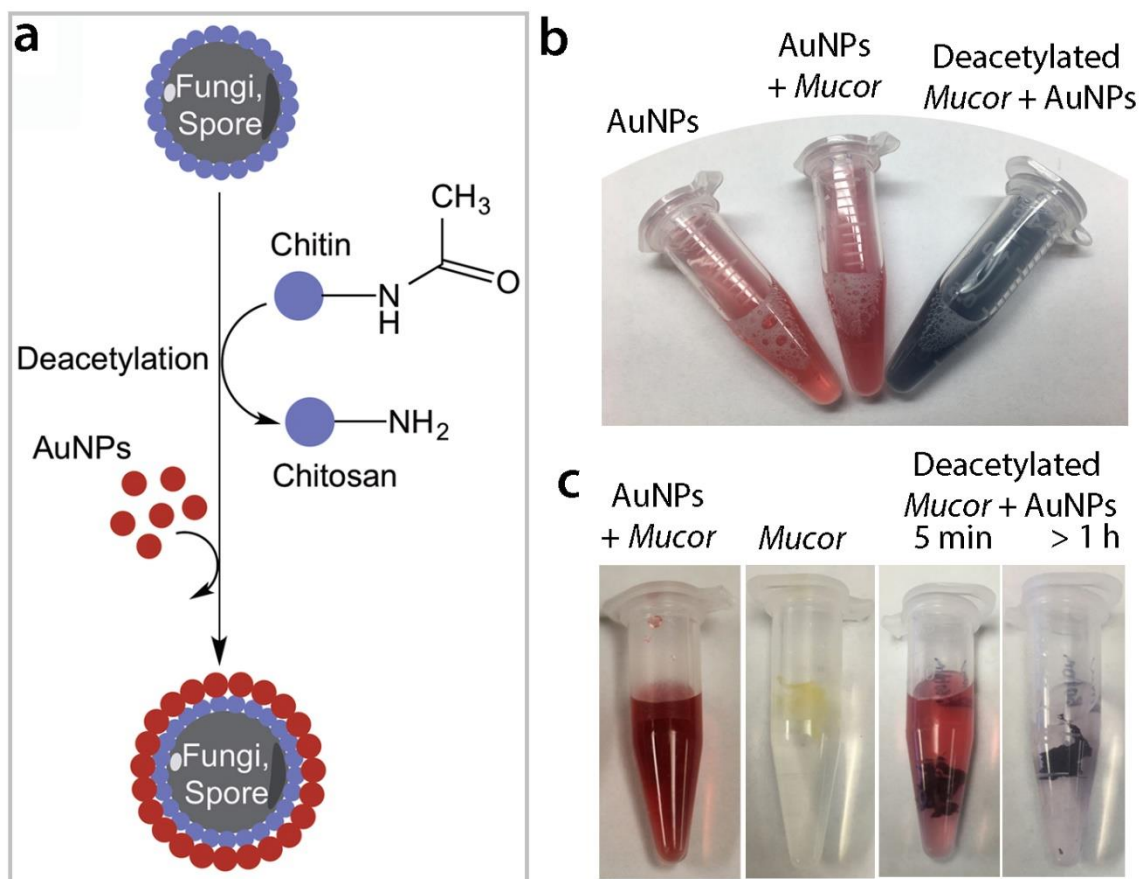


Figure 2. Plasmonic cell nanocoating using chitin. **(a)** Scheme of plasmonic cell nanocoating of fungi by converting chitin layers into chitosan through deacetylation. **(b)** Pictures of AuNP solution mixed with fungi (*Mucor*), showing the change in color after fungi deacetylation by 50% NaOH. When a large piece of fungi is used **(c)**, the yellowish substance (*Mucor*) turns dark by assembling the nanoparticles on its surface after 5 min. The solution becomes transparent once all the nanoparticles are assembled on the fungal surface. The microbial concentrations used in all these pictures are at least 10^8 cfu.mL⁻¹.

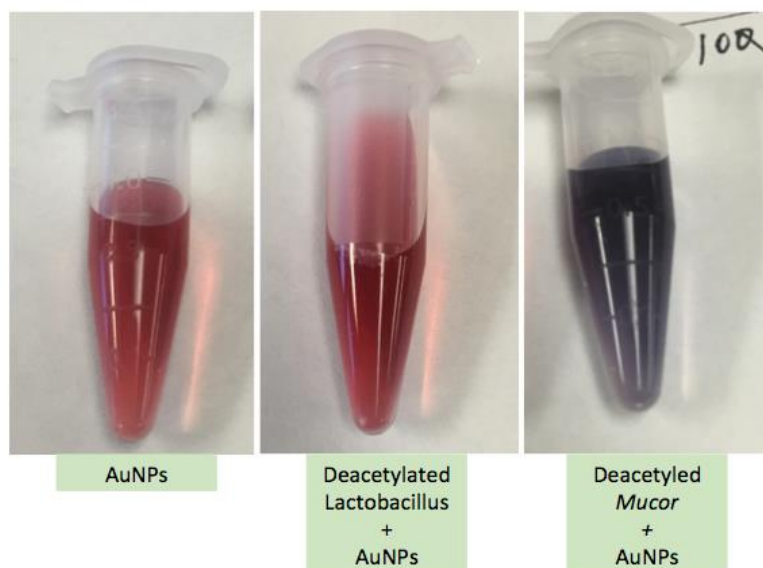


Figure 3. Effect of the deacetylation *Lactobacillus delbrueckii* and *Mucor circinelloides* on the assembly of gold nanoparticles.

To confirm that the color shift (*i.e* nanoparticle assembly) in the microbial solutions is the result of cell nanocoating, bacterial and fungal samples were prepared and analyzed by scanning electron microscopy (SEM), transmission electron microscopy (TEM) and energy-dispersive X-ray spectroscopy (EDX). As shown in **Figure 4**, cell nanocoating can be clearly observed in all microorganisms. Additional SEM images are shown in **Figure 5** and TEM images are presented in **Figure 6**. It is important to note that it was relatively difficult to obtain some of the SEM and TEM images of nanocoated cell. While fungal deacetylation results in highly stable nanocoating due to the covalent bonding of chitin to other components in the fungal cell walls, bacterial reduction yields less stable samples. In fact, the Dsbc protein layers seem to easily peel-off after nanocoating, suggesting non-covalent bonding of Dsbc proteins layers to the bacterial cell wall (**Figure 7 and Figure 8**). The images of nanocoated *Lactobacillus delbrueckii*

were successfully obtained only after reducing the pH of the solution to 4, which likely strengthened electrostatic interactions between the Dsbc proteins and the bacterial cell wall. This challenging experiment reminds of the difficulty of observing and imaging microbial S-layers.

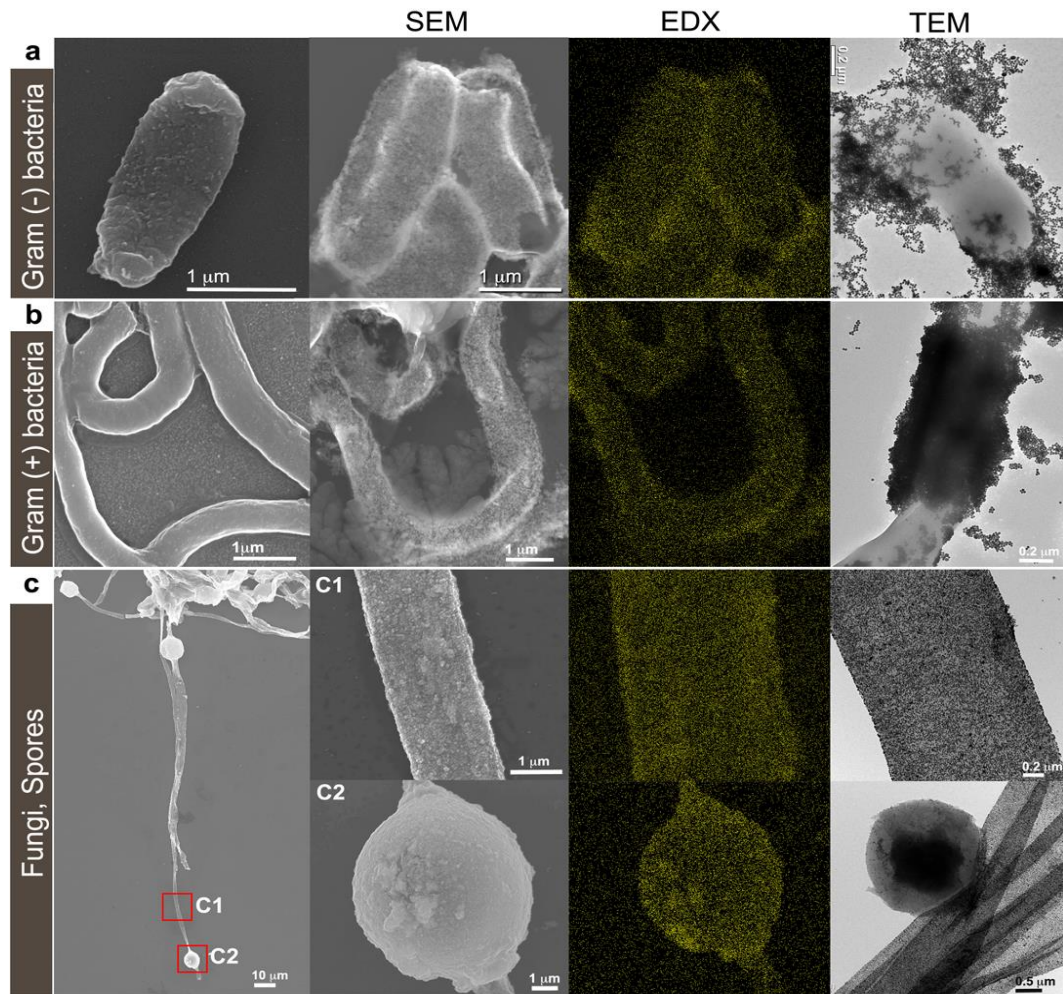


Figure 4. Scanning and transmission electron microscopy imaging (SEM, TEM) and energy-dispersive X-ray spectroscopy mapping (EDX) of microbial cells coated with gold nanoparticles. The images depict cell nanocoating of (a) *E. coli* (gram-negative bacteria), (b) *Lactobacillus* (gram-positive bacteria), (c) *Mucor circinelloides* (fungi). The yellow patterns in the EDX mapping images shows the presence of gold and reveals the distribution of gold nanoparticles on the surface of microorganisms. The *E. coli* and *Lactobacillus* images were obtained after adjusting the pH of the reaction solution to 4.

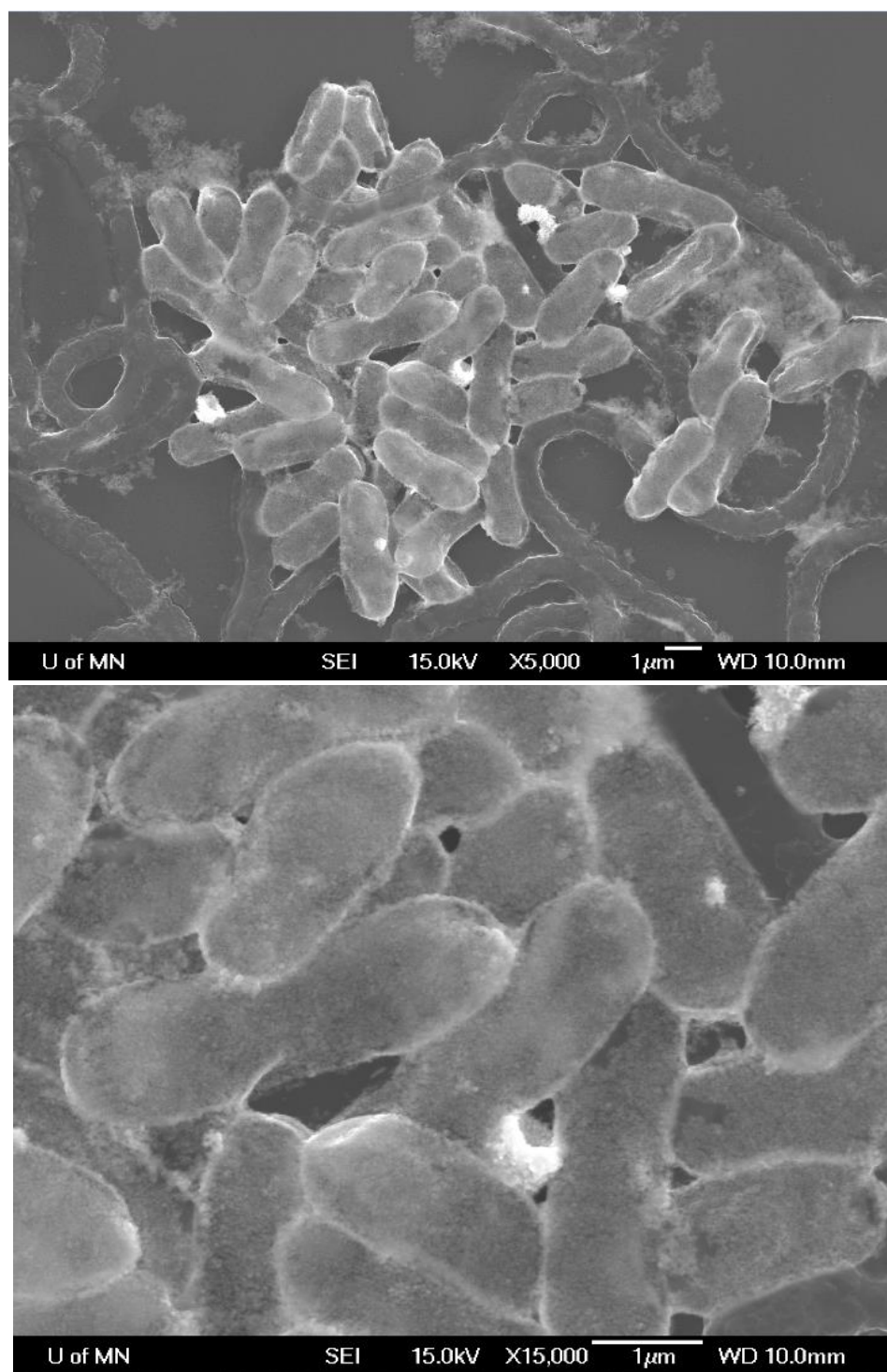


Figure 5a. SEM images of AuNP-coated *E. coli* (top) and at higher magnification (bottom)

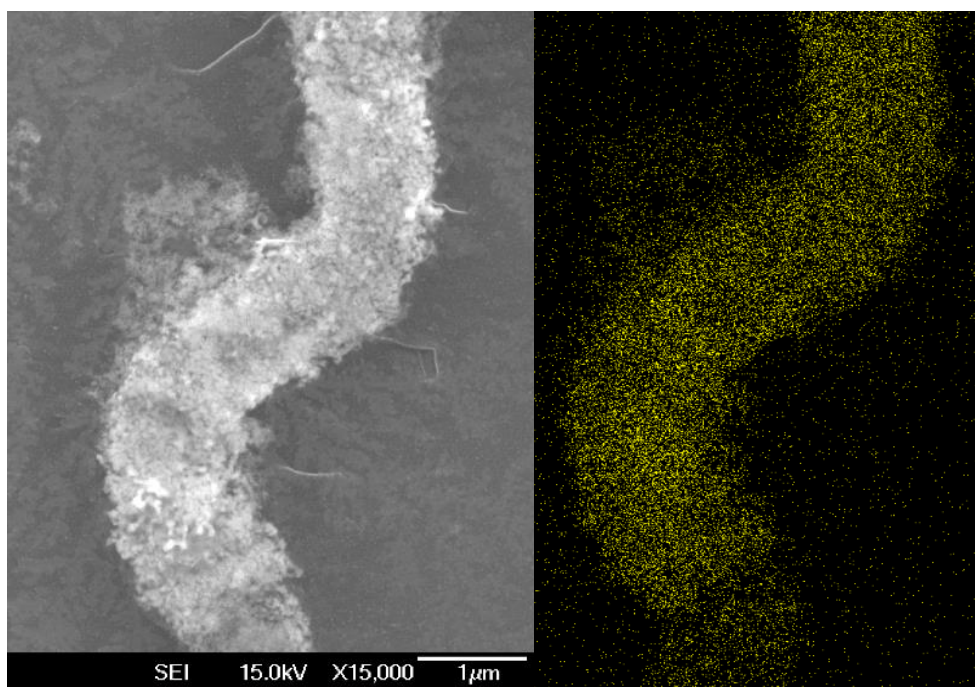


Figure 5b. SEM images of AuNP-coated *Lactobacillus* (left) and EDX image of Au mapping (right)

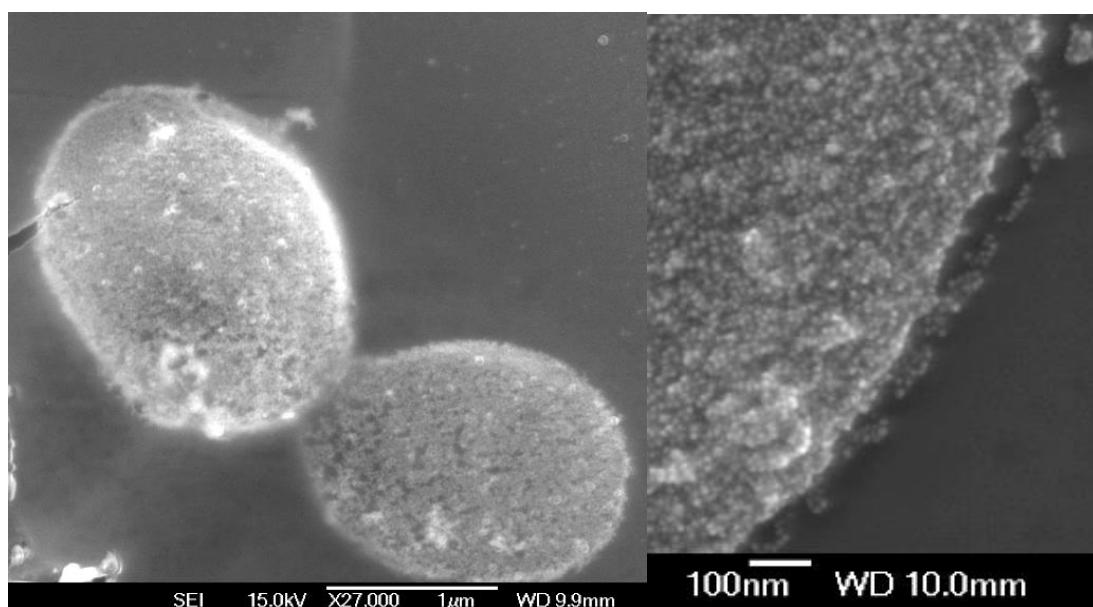


Figure 5c. SEM images of AuNP-coated *Mucor circinelloides* (left) and at higher magnification(right)

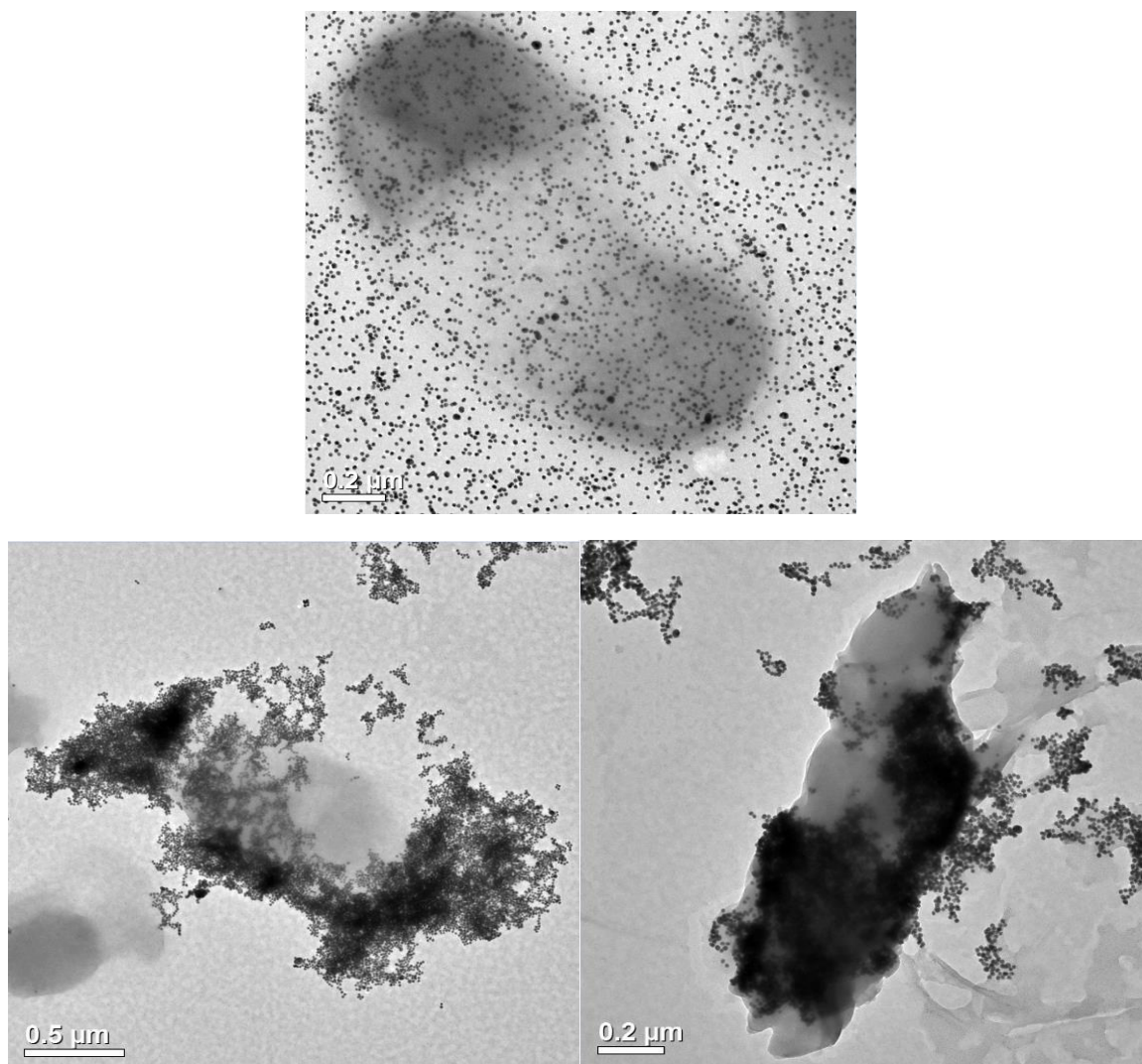


Figure 6a. TEM images of AuNP-coated *E. coli* nanocoating, *E. coli* with AuNPs before the addition of TCEP (top) and after coating (bottom left and right)

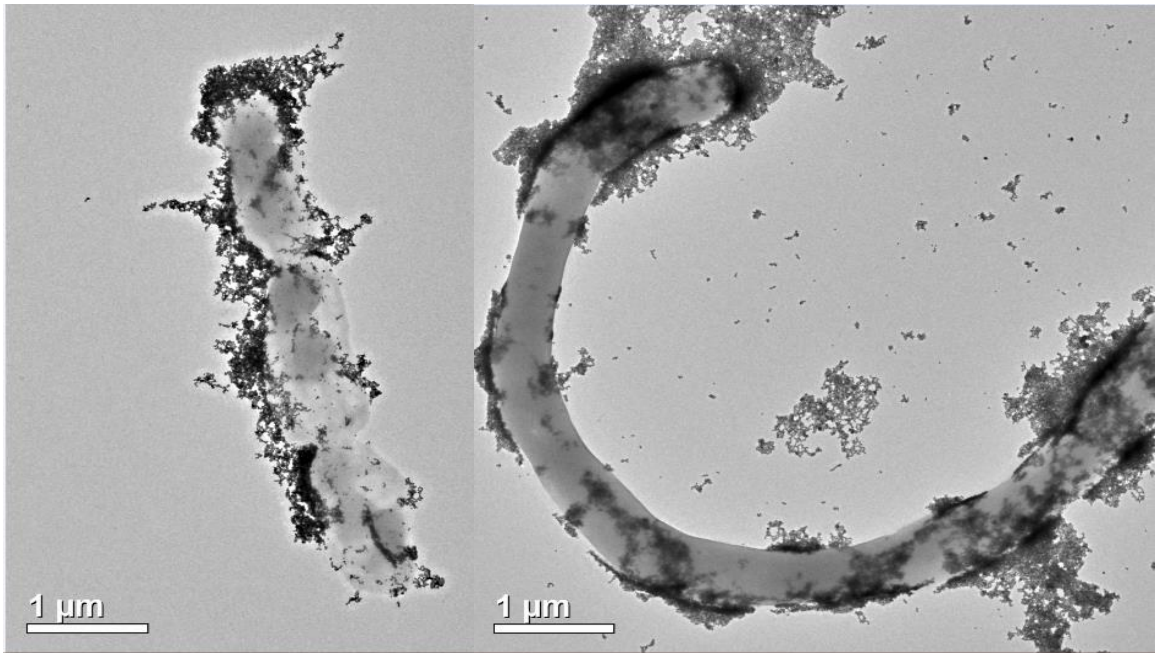


Figure 6b. TEM images of AuNP-coated *Lactobacillus delbrueckii*

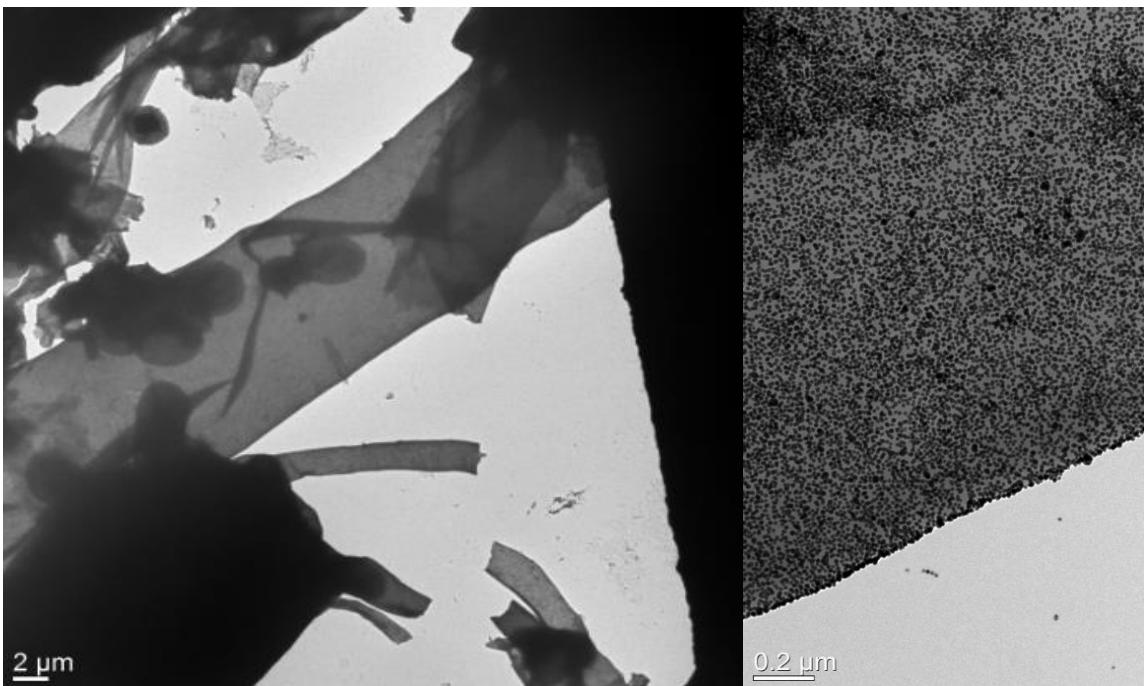


Figure 6c. TEM images of AuNP-coated hyphae and spores of *Mucor circinelloides*.

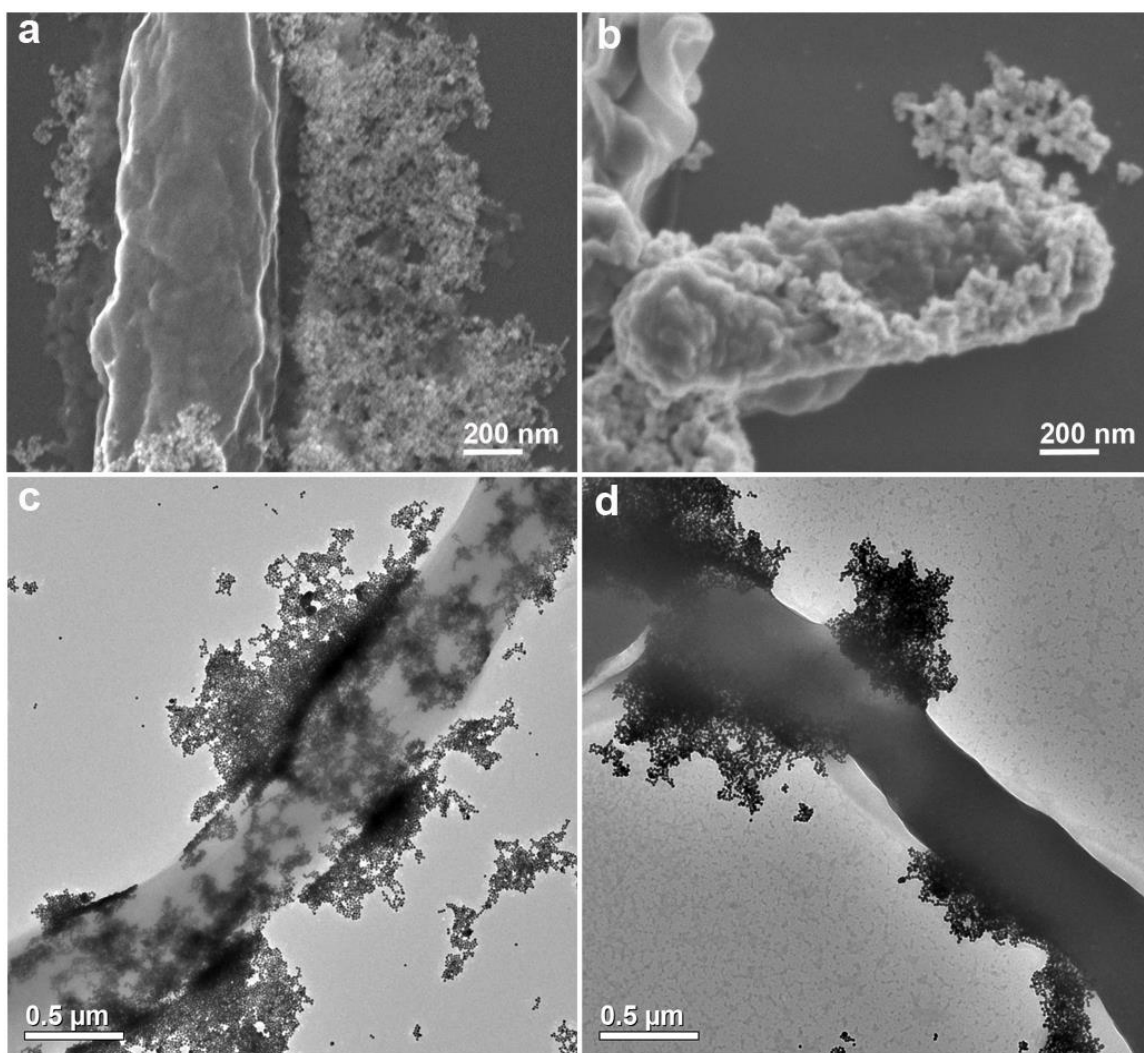


Figure 7. Stability of the microbial Dsbc surface protein layers. SEM (a, b) and TEM (c, d) images showing nanoparticle layers attached and peeled-off the surface of bacteria *Lactobacillus* (a, c, d) and *E. coli* (b) after cell nanocoating with gold nanoparticles.

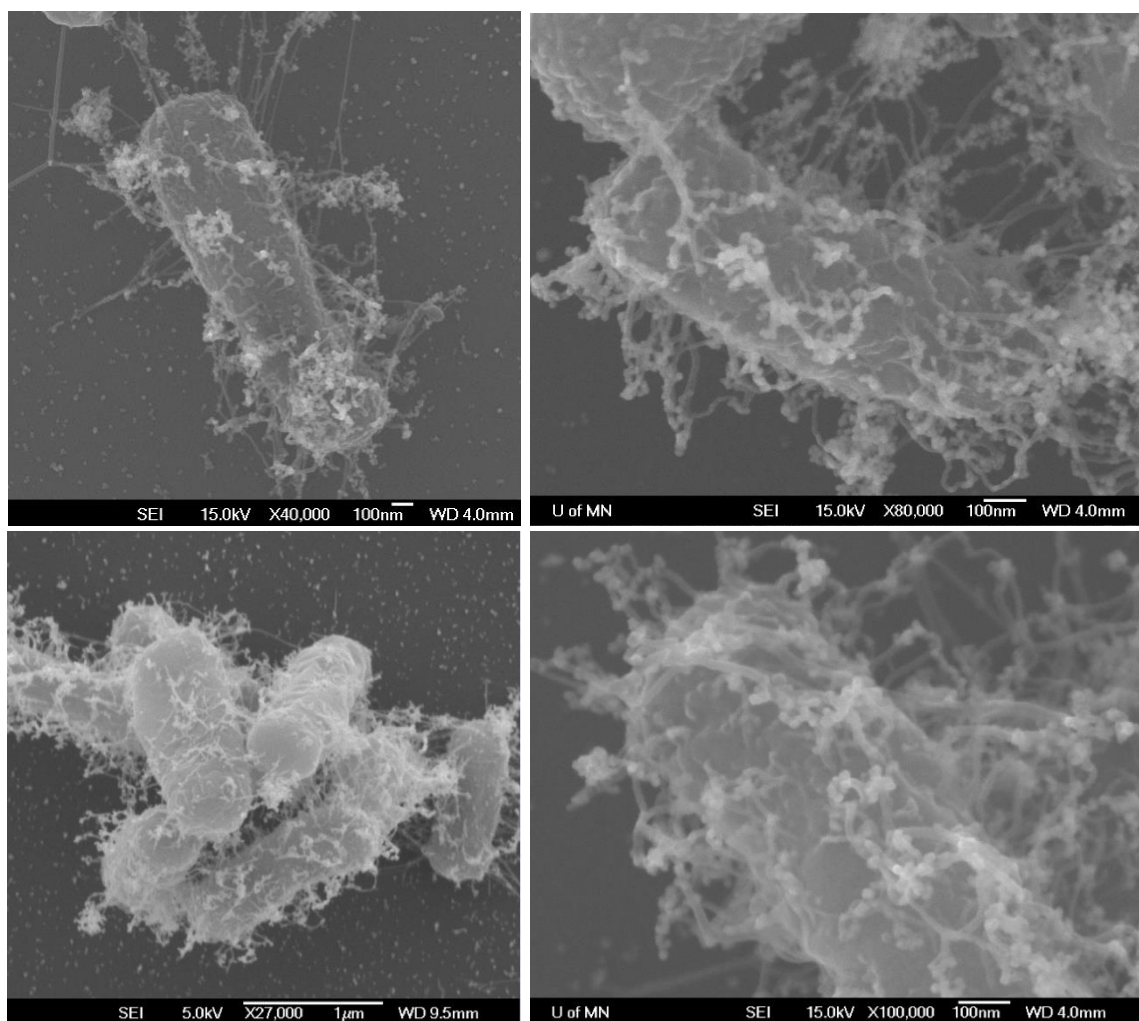
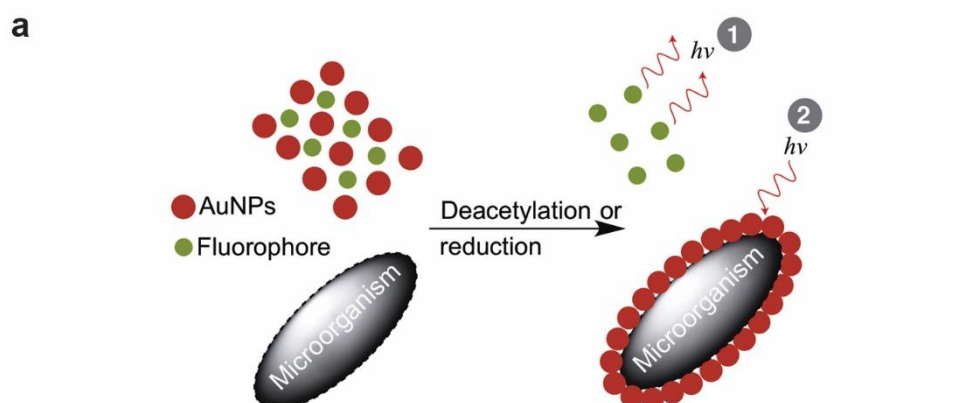


Figure 8. SEM images of bacteria *E.coli* showing the binding of AuNPs to bacterial pili.

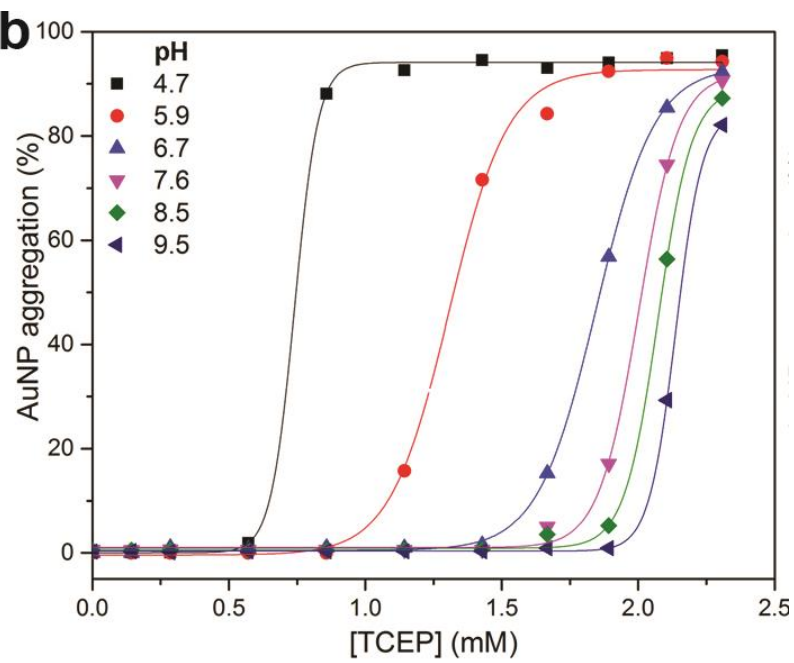
4.3. Plasmonic and fluorescence detection assays

The assembly of AuNPs on the microbial surface can be used for microbial detection by monitoring a change in absorbance due to localized surface plasmon resonance. It can also be achieved by monitoring the change in fluorescence quenching of an aqueous fluorophore due to the presence of AuNPs (**Figure 9a**). Since nanoparticle assembly is caused by reduction or deacetylation of the microbial surface layers using a reducing agent, it is important to first investigate the interaction of the reducing agent with both the nanoparticles and microorganisms. It is also important to know the effect of the pH conditions on these interactions. Different concentrations of two reducing agents; TCEP and 2-mercaptoethanol (BME) have been used at different pH to evaluate the impact of both parameters on the interaction. **Figure 9b, 9c** show that concentrations below 1 mM for TCEP and over 100 mM for BME are suitable for detection assays at a pH around 6.5. The working concentration range for the reducing agent is a function of the pH conditions. Outside that range, assembly may be caused solely by the reducing agent by changing the zeta potential and disrupting the electrostatic repulsion between AuNPs. Because of its irreversible interaction with the disulfide bonds, TCEP is used here for the rest of the experiments. Since TCEP is not regenerated during the reaction, higher concentrations may be used for faster reduction of the microbial surface. Increasing the pH of AuNP allows for an increase in TCEP concentration without causing nanoparticle assembly. However, the pH cannot be increased over 8 since the free thiol groups on the microbial surface will be deprotonated at higher pH (Nair et al., 2014), which hinders their interaction with the AuNPs.

a



b



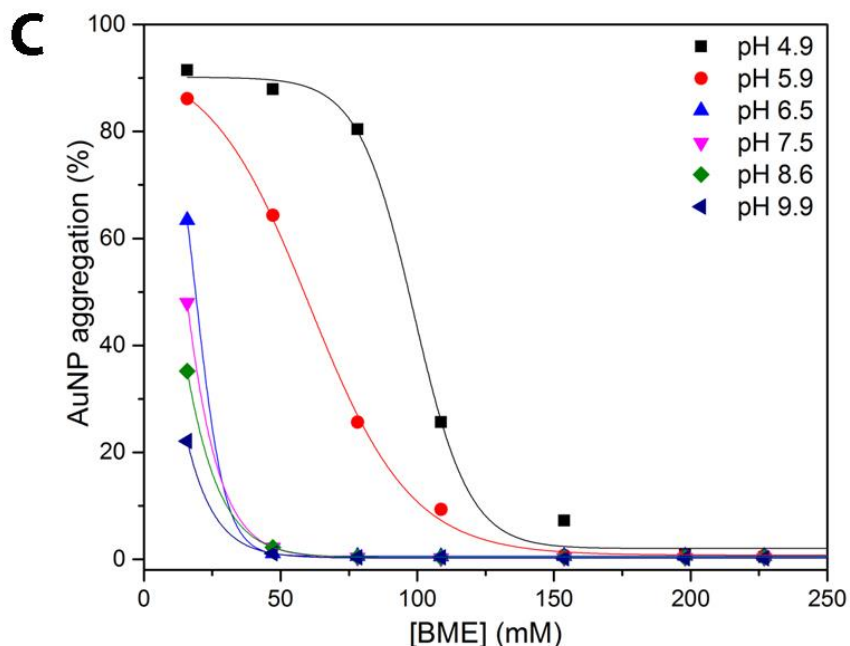
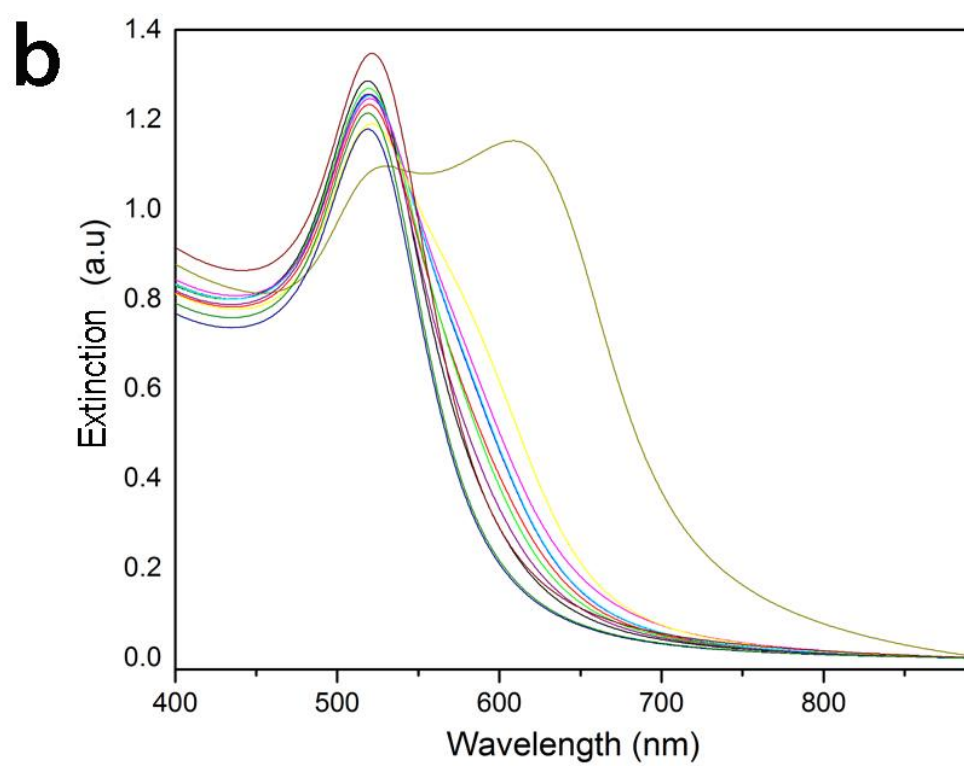
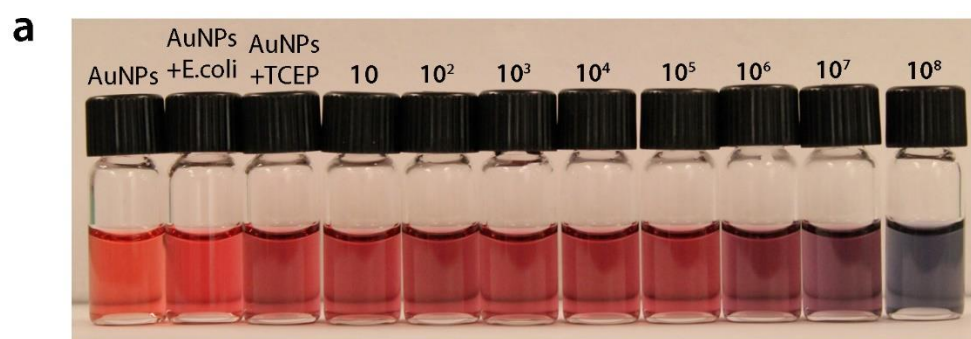


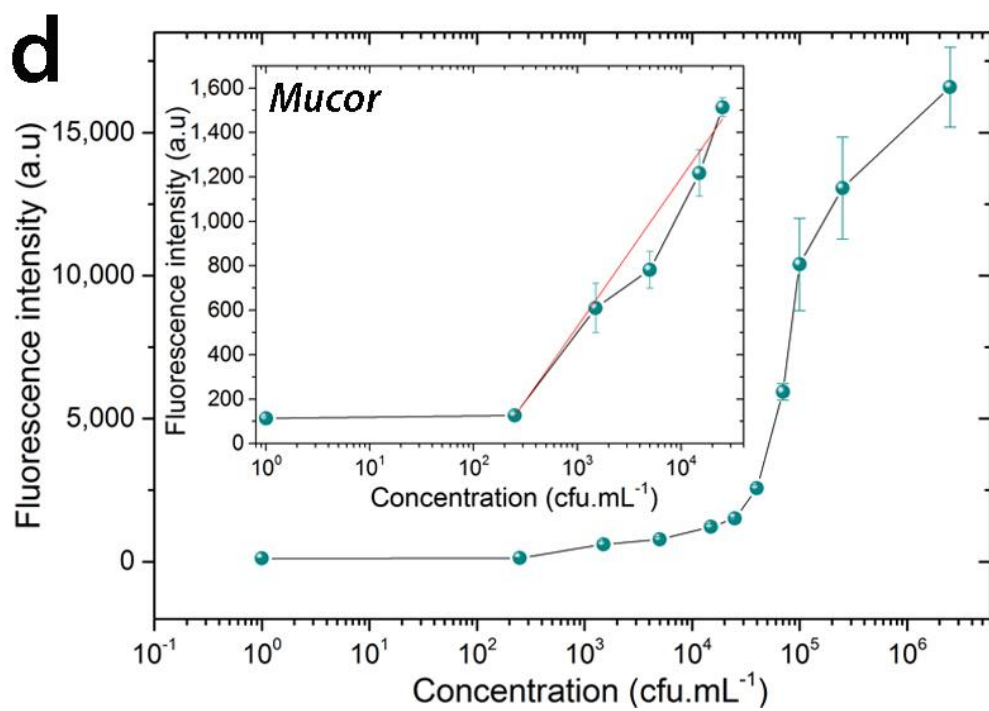
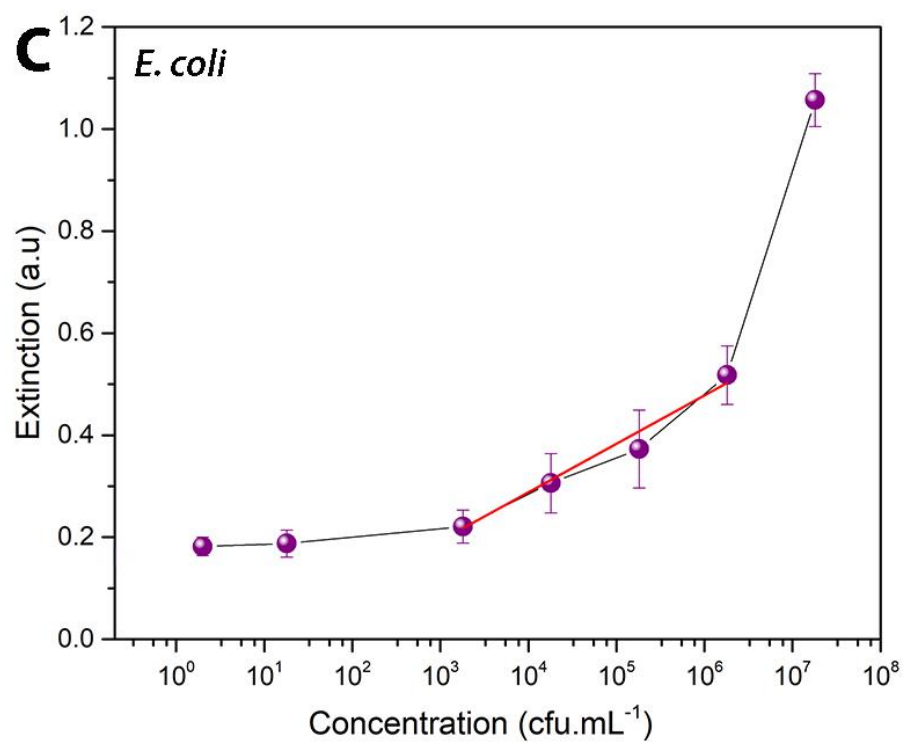
Figure 9. (a) Scheme depicting the concept of homogenous rapid microbial screening assays based on plasmonic cell nanocoating. The detection can be performed either with (1) fluorescence emission and quenching by AuNPs or (2) light absorption by plasmonic coupling of localized surface plasmon resonance on AuNPs. (b) Effect of the reducing agent TCEP concentration on the AuNPs stability and aggregation at different pH conditions. (c) Effect of the reducing agent BME concentration on the AuNPs stability and aggregation at different pH conditions. The concentrations that do not cause nanoparticle aggregation are used for microbial screening assays

As depicted in **Figure 10a**, microbial concentrations down to 10^5 cfu.mL⁻¹ can induce a color change visible to the naked eye. The concentration of the nanoparticles may be reduced so that there will be no single nanoparticle left in solution after coating the cells, thus providing a better color shift for naked eye assessment (**Figure 11**). However, the zeta potential of the diluted AuNP solution should be kept the same as the original solution by diluting the nanoparticles in a citrate solution. To assess the limit of detection of the concept using localized surface plasmon resonance spectroscopy, different concentrations of microbial suspensions ranging from 10 to 10^8 cfu.mL⁻¹ were

used for the assay (**Figure 10**). The absorption peak at around 616 nm (corresponding to assembled AuNPs) was analyzed with UV-visible spectroscopy, and cell concentration was verified using flow cytometry. The correlation between the peak intensity and the microbial concentration reveals a detection limit at 1500 cfu.mL⁻¹ for *E. coli* (**Figure 10c**). Since the AuNPs assemble on the microbial surface, larger microorganisms are expected to have lower detection limits.

To demonstrate the versatility of the detection concept, the same experiments were performed using fluorescence spectroscopy (**Figure 10d**). In this case, AuNPs were mixed with a fluorophore, *i.e* Rhodamine 6G, and added to a microbial suspension. The presence of the AuNPs in solution quenches the fluorescence emission. The addition of TCEP and the subsequent assembly of the nanoparticles around the microorganisms leave the fluorophore alone in solution, leading to the enhancement of the fluorescence signal. The correlation between the microbial concentration and the fluorescence signal reveals a limit of detection at 35 cfu.mL⁻¹ for *E. coli* and at 1500 cfu.mL⁻¹ for *Mucor circinelloides* (**Figure 10e**) (Armbruster & Pry, 2008). Both absorption and fluorescence transduction methods were performed on environmental samples, which were collected by swabs on a contaminated surface and suspended. Similarly, to any other rapid microbial detection technique, the implementation of the concept to more complex samples requires upstream technologies to isolate the microorganisms from the matrix. Future work will focus on using surface markers to selectively target gram-positive or gram-negative bacteria.





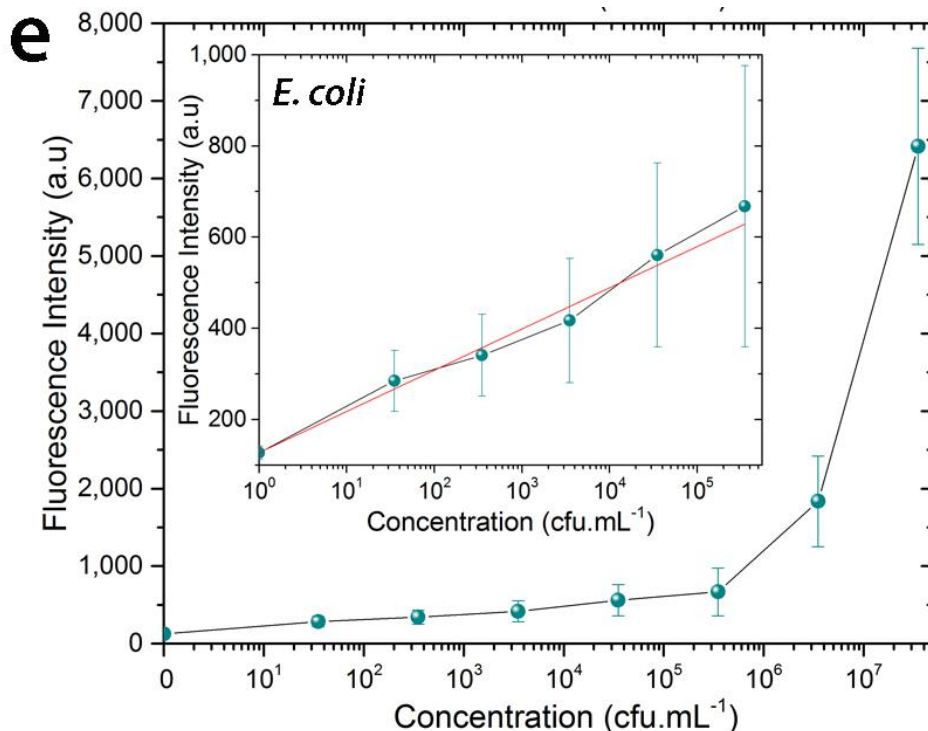


Figure 10. Rapid microbial screening of bacteria and fungi by plasmonic cell nanocoating. (a) Image of AuNP solution with different concentrations of bacteria *E. coli*. The image was taken 2 min after mixing the nanoparticles with reduced bacteria. (b) UV-visible spectra of the different samples showed in Figure (a). Single nanoparticle solution exhibit a maximum absorption peak at around 525 nm. During cell nanocoating, a second peak appears and grows in intensity at around 616 nm. This peak is caused by plasmonic coupling of assembled gold nanoparticles, and reflects the deposition of the nanoparticles on the microbial surface. (c) The correlation between the microbial concentration in Figure (a) and the absorption intensity. A linear range can be identified between 10^3 and 10^7 cfu.mL⁻¹ ($R^2=0.98$). (d) Detection of fungi *Mucor* using fluorescence quenching of Rhodamine 6G. (e) Detection of bacteria *E. coli* using fluorescence quenching of Rhodamine 6G. The linear ranges are from 250 to 10^4 cfu.mL⁻¹ for *Mucor* ($R^2=0.98$), and 0 to 10^5 cfu.mL⁻¹ for *E. coli* ($R^2=0.99$).

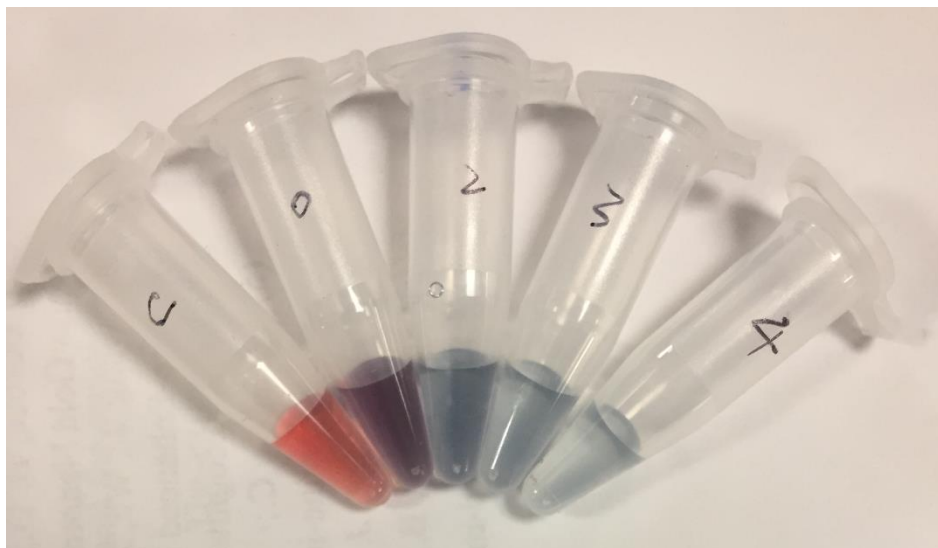


Figure 11. Effect of the reduction of the concentration of the gold nanoparticles on the visual reading of the microbial load. The tube labeled “c” is a control (AuNP solution). The numbers on the tubes labeled 0, 2, 3 and 4 represent the dilution factor of the gold nanoparticle solution used for interaction with the microorganisms. Except the control, all tubes contain *E.coli* at a concentration of 10^8 cfu.mL⁻¹.

CHAPTER 5 Conclusions and Prospective

Conclusion

In summary, this work reports a new concept for rapid microbial screening using specific cell nanocoating. By targeting surface molecules on the microbial surface, gold nanoparticles can coat the surfaces of the microorganisms and allow different transduction systems to be used for further quantification. The study suggests the existence of reducible surface molecules on the surfaces of different microorganism with high affinity to gold, likely disulfide bond-containing surface proteins on the microbial surface and chitin molecules on the fungal surface. The presence of such molecules has been further confirmed and validated for all 20 microorganisms studied, which includes gram-positive bacteria, gram-negative bacteria, yeasts, molds and dimorphic fungi. The results show that plasmonic cell nanocoating using either Dsbc proteins for microbial screening or chitin for fungal screening enables a highly sensitive detection, within 5 and 30 min respectively. The detection can be performed with the naked eye (LOD: $10^5 - 10^6$ cfu.mL⁻¹), using UV spectrophotometer or using a portable fluorometer (LOD: 35 – 1500 cfu.mL⁻¹).

Prospective

As the government agencies and legislations are developing new standards for microbial control, rapid, cheap and accurate tools for microbial detection are in great need by the market. In this study, a rapid detection system is demonstrated mainly for the application in rapid microbial diagnostics. Although the assay is not intended for selective detection, its advantages lie in simplicity, rapidity, and effectiveness in both

qualitative and quantitative evaluation of the total microbial load. Compared to the current commercial assays in the market, its quickness and low limit of detection can be potentially used by healthcare providers and environmental or food inspection agencies. In the future, this system can be combined with a microbial separation system to allow real-world-sample and real-time diagnostics for surface hygiene screening, foodborne pathogen screening and water quality control. In addition, other transduction methods or control techniques could be envisioned by exploiting the optical, electrical, thermal or catalytic properties of gold nanoparticles or other thiol-reactive components.

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